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EFFECT OF NON-THERMAL PLASMA TECHNOLOGY ON MICROBIAL INACTIVATION AND TOTAL PHENOLIC CONTENT OF MODEL LIQUID FOOD AND BLACK PEPPER GRAINS

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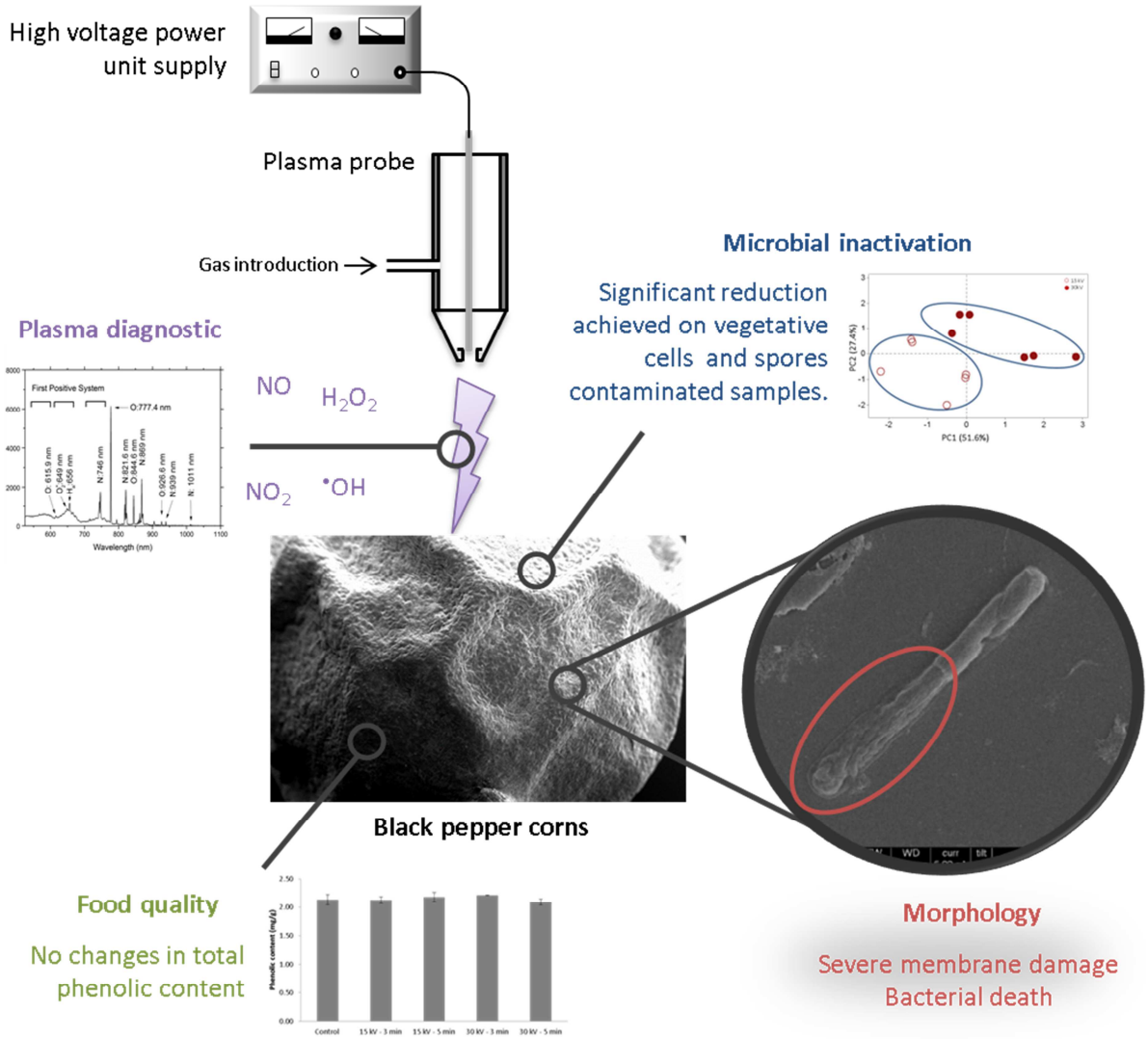
Graphical abstract



Abstract

The objectives of this study were to investigate the effects of cold plasma technology on the growth and survival rates of vegetative cells and spores, and total phenolic content of black pepper grains. Plasma treatment was carried out using a non-thermal plasma jet system operating at 20 kHz using atmospheric air at a flow of 11 L/min. Two matrices were used, a model liquid food system and black pepper grains, both inoculated with *Bacillus subtilis* vegetative cells and spores. The samples were treated at 15 and 30 kV for 3 - 20 min. The plate count method was used to observe the colony-forming units at selected storage times i.e. at 1, 24 and 48 h post treatment at 4 ^{o}C. The highest log reduction was observed at 24 h post treatment, i.e. 2.92 log reduction. A 1 log reduction was achieved in the case of black pepper inoculated with spores for all selected storage times. No significant differences in total phenolic content were observed between treated and non-treated samples ($p > 0.05$). Optical emission spectroscopy was used to detect reactive species which could be responsible for cell death. Atomic oxygen, atomic nitrogen, hydroxyl radicals, nitrite oxide and nitrate were detected in light emitted from the plasma. Cell membrane damage caused by non-thermal plasma technology was observed using scanning electron microscopy. This study concludes that cold plasma technology has potential for industry application in food processing to reduce microbial loads in dried foods with limited impacts on food quality.

Keywords: Non-thermal plasma; microbial inactivation; optical emission spectroscopy; food quality; dried ingredients.

1. Introduction

Dried food ingredients including grains, spices and powders have low water activities which limits microbial growth. However, pathogenic and spoilage microorganisms and spores have been found in several dried foods. For instance, a Salmonella outbreak associated with the consumption of ready-to-eat salami products containing contaminated black pepper has been reported in the United States (Gieraltowski, Julian, Pringle, Macdonald, Quilliam, Marsden-Haug, et al., 2013). The contamination event generally occurs because of poor hygiene practices during cultivation, harvesting and food manufacturing. A significant number of microorganisms is associated with dried food ingredients, pathogens of particular significance include *Salmonella* spp, *Bacillus* spp and *Clostridium perfringens*. In response to adverse environmental conditions, the last two microorganisms have the ability to produce dormant structures or bacterial spores, which are resistant to many treatments. Conventional technologies for decontamination of dried ingredients include super-heated steam, fumigation with ethylene or propylene oxide and ionizing radiation. However, there are many limitations associated with use of these technologies including nutrient degradation, health risks, legislative obstacles and consumers' concerns. Indeed, ethylene and propylene oxide have been banned in the European Union because of carcinogenic and mutagenic concerns (Fowles, Mitchell, & McGrath, 2001; Regulation, 2008). Even though gamma-rays and X-rays have been shown to have strong potential to help ensure food safety with limited impacts on quality, consumers continue to have a negative perception of food irradiation treatments (Bearth & Siegrist, 2019). Additionally, conventional thermal treatments e.g. super-heated steam affects food quality due to the temperatures involved in the process. These limitations have encouraged research to investigate alternative approaches to ensure safety of dried food ingredients.

Plasma is defined as a partially or wholly ionized gas composed of positive and negative ions, electrons, photons, free radicals and neutrons atoms and molecules (Fridman & Kennedy, 2004). The ionization of these chemically reactive components can occur upon exposure to different energy sources, the most common being thermal, microwave and radio frequency, radioactive (gamma radiation) and X-ray electromagnetic radiation. Depending on its thermodynamic equilibrium state, plasma can be classified as thermal or non-thermal. Unlike in thermal plasma, the electrons and other gas components comprising cold plasma exist in a non-thermodynamic equilibrium (Shashi K Pankaj & Keener, 2017). Cold plasma has traditionally mainly been used in the bio-medical, textile and polymer industries (Fanelli & Fracassi, 2017; Kim, Kim, Hong, & Yang, 2010; Sun & Stylios, 2004). However in the last decade it has been increasingly investigated as an alternative tool to the conventional technologies in the food industry. Advantages of cold plasma compared to traditional technologies include: extension of product shelf life with limited impacts of food quality, higher retention of the nutrients in the food matrix, and reduced environmental impacts due to lower energy, water and solvent requirements (Hati, Patel, & Yadav, 2018;

Shashi K Pankaj, Wan, & Keener, 2018). Applications of cold plasma technology in the food industry investigated to date include inactivation of microorganisms in food products (Dasan, Yildirim, & Boyaci, 2018; Misra & Jo, 2017; Pasquali, Stratakos, Koidis, Berardinelli, Cevoli, Ragni, et al., 2016), modification of functional properties of food matrices (Bahrami, Bayliss, Chope, Penson, Perehinec, & Fisk, 2016; Kovačević, Putnik, Dragović-Uzelac, Pedisić, Jambrak, & Herceg, 2016; Thirumdas, Saragapani, Ajinkya, Deshmukh, & Annapure, 2016), improvement of food packaging (Oh, Roh, & Min, 2016; Shashi Kishor Pankaj, Bueno-Ferrer, Misra, Milosavljević, O'Donnell, Bourke, et al., 2014) and hydrogenation of edible oils (Yepez & Keener, 2016). Adaptive sources and designs employed these applications include: dielectric barrier discharges, corona discharge, gliding arc discharges, radio frequency, microwave and jet plasma (Ekezie, Sun, & Cheng, 2017). The energetic species generated by plasma can alter and inactivate microorganisms (Misra, Tiwari, Raghavarao, & Cullen, 2011). This paper describes the effect of a non-thermal atmospheric pressure plasma jet on the microbial loads of contaminated liquid model food and black peppers grains, and on the total phenolic content of black pepper.

2. Materials & Methods

2.1 Dried ingredients

Black pepper grains (*Piper nigrum* L.) were purchased from a commercial supplier (Brand Heera, P&B Ltd, West Yorkshire, United Kingdom).

2.2 Microbial growth and inoculation

Two matrices were used, a liquid model food system inoculated with *Bacillus subtilis* vegetative cells, and black pepper grains inoculated with *B subtilis* vegetative cells and spores. *Bacillus subtilis* DSM 618 (Merck KGaA, Germany) was grown in nutrient broth for 24 h at 30 ^{o}C under shaking conditions (150 rpm). Black pepper grains were inoculated with the culture solution at 10^{7} CFU/mL at a ratio of 1:2 (w:v) and submitted to gentle agitation (150 rpm) at 30 ^{o}C for 2 h. A solution of 10^{5} CFU/mL of *B subtilis* spores (Merck KGaA, Germany, Product number 1.10649.0001) was heated at 80 ^{o}C for 10 min to inactivate potential vegetative cells. The dried ingredients were then inoculated with the spore suspension following the same protocol outlined above. After inoculation, the samples were dried at 35 ^{o}C for 90 min. The treatment was conducted as described in Section 2.3. Experimental tubes were closed directly after treatment and stored at 4 ^{o}C until analysis. The microbial population was evaluated at 1, 24 and 48 h post treatment as follows: 1 g of sample was re-suspended into maximum recovery diluent (peptone saline broth (PBS)) in order to obtain 1-

fold dilution. Decimal dilutions were carried out and spread on nutrient agar plates. After 24 h at 30 ^{o}C, the CFU was counted. All experiments were replicated three times.

2.3 Plasma system and conditions

Experiments were carried out using a non-thermal atmospheric pressure plasma jet (APPJ) system (Figure 1) sourced from the National Centre for Plasma Science and Technology at Dublin City University (Glasnevin, Dublin 9, Ireland). The APPJ consisted of a variable high voltage power supply unit at a frequency of 20 kHz, operating with a gas flow rate of 11 L/min with air as the working gas. Plasma was generated between two stainless steel electrodes, an outer conical shaped ground electrode and inner pin shaped electrode connected to the power supply. The plasma jet diameter was 30 mm. Plasma generated within this region exited through a small gap in the ground electrode entering ambient conditions in the plume region where it came into contact with treatment samples.

5 g of each sample in sterile glass tubes were placed at 5 cm below the plasma probe. The samples were treated at 15 and 30 kV for 3 and 5 min if inoculated with vegetative cells, and for 10 and 20 min if inoculated with spores. The experiments were carried out at room temperature.

2.4 Scanning electron microscopy

A scanning electron microscope was used to investigate the effect of plasma treatment on the morphological characteristics of the *B subtilis* vegetative cells. Cells preparation was carried out as described by Fratesi, Lynch, Kirkland, and Brown (2004) with slight modifications and was composed of three steps: overnight fixation using 2.5 % glutaraldehyde (G5882 – Sigma Aldrich) in 0.1 M sodium phosphate buffer (PBS, pH 7.2) at 4 ^{o}C; dehydration (after five washing steps with PBS) using a series of ethanol concentrations (50, 60, 70, 80 and 90 % for 5 min and 100 % for 15 min two times); and drying using a graded Hexamethyldisilizane series (HMDS, 440191 – Sigma Aldrich) of 33, 50, 66 % for 15 min and 100 % for 15 min two times. The samples were then mounted on stubs using double-sided carbon tape, and sputter coated with gold, using an Emitech K575X Sputter Coating Unit, to prevent surface charging by the electron beam. Samples were examined using a FEI Quanta 3D FEG DaulBeam (FEI Ltd, Hillsboro, USA). The entire surface of the sample was examined, and representative images of the sample were taken.

2.5 Total phenolic content (TPC)

In order to determine if non-thermal plasma technology has an impact of food quality, total phenolic content (TPC) which is a key quality parameter of black pepper was analysed before and after treatment. Total phenolic content was determined using the Folin - Ciocalteu reagent (FCR) assay as outlined by Singleton, Orthofer, and Lamuela-Raventós (1999) with slight modifications as described by Rössle, Wijngaard, Gormley, Butler, and Brunton (2009). The concentration of TPC was expressed in mg Gallic acid equivalent (GAE) per g of dry weight of sample. All measurements were carried out in triplicate.

2.6 Optical emission spectroscopy

Optical emission spectroscopy (OES) measurements were made using an Ocean Optics HR2000+ spectrometer, over a spectral range of 190 – 1100 nm. To record the spectrum, high speed acquisitions of the plasma jet were made, with an acquisition time of 100 ms. Each acquisition consisted of five scans over the spectral range, averaged over five acquisitions.

2.7 Statistical analysis

All experiments were carried out in triplicate and average values reported. Analysis of Variance (ANOVA) and separation of means was carried out using SAS (Version 8.0) and Principal Component Analysis (PCA) was performed using Minitab (Version 16.0). Treatment means were separated using Tukeys' test and considered significantly different at $p < 0.05$. Score plot analysis with graphs containing the first two principal components was carried out.

3. Results & Discussion

3.1 Microbial inactivation

Figure 2a-c shows the vegetative cells population (log CFU/mL) in a model food system and inoculated black pepper for 15 kV and 30 kV for a treatment time of 3 and 5 min respectively after storage for 1h (Fig 2a), 24 h (Fig 2b) and 48 h (Fig 2c) post treatment at 4 ^{o}C. For all the samples, a significant difference ($p < 0.05$) was observed after storing for 24 h compared to control or 1 h post treatment. For 3 min of treatment at 15 kV, the microbial loads of the model food was reduced from 7.27±0.07 log CFU/mL to 6.48±1.30 log CFU/mL after 1 h of storage, and 3.65±0.23 log CFU/mL and 3.42±0.35 log CFU/mL after 24 and 48 h of incubation respectively. However, the results for the samples stored for 24 and 48 h did

not show any significant difference ($p > 0.05$). Vegetative cells population inoculated in black pepper grains showed higher reductions at increased voltage and treatment time. Storage of samples for 48 h did not show any significant decrease in the microbial population inoculated in black pepper. For example, vegetative cells population inoculated in black pepper was significantly reduced to 1.63 log CFU/mL after treatment at 30 kV for 5 min after 1 h incubation and to 1.74 log CFU/mL after 48 h storage at comparable conditions. Figure 3 presents the log reductions of samples contaminated with spores. No significant reductions ($p > 0.05$) in spores were observed for treatment times < 10 min (data not reported) whereas significant reductions were observed at 10 and 20 min treatment time for both 15 kV and 30 kV compared to control. No significant decrease in spores population was observed during storage for 24 and 48 h at 4 ^{o}C ($p > 0.05$). However, a significant reduction of 1.16±0.98 log (CFU/g) was reached for the spores contaminated black pepper grains treated at 15 kV for 3 min at 1 h post-treatment ($p < 0.05$).

This study shows plasma efficiency peaks one day after treatment, and it is not required to extend the storage period. No significant effect was observed for treatment time ($p > 0.05$), but the different voltage levels used showed a significant difference ($p < 0.05$). Scatter plot showing similarities and differences between voltage and treatment time analysed using Principal Component Analysis (PCA) is presented in Figure 4. PCA was able to explain 100 % of data variability by two principal components (PC1 (62.6 %) and PC2 (37.4 %)). We can observe that the first component PC1 has been effective in separating the different voltages used (15 and 30 kV) and treatment times (3 and 5 min). Comparing both food matrices inoculated with *B subtilis* vegetative cells, it can be observed that plasma has a higher effect on the model liquid systems compared to black pepper. Plasma affects the surface of food products, and the roughness and cavities of black pepper grain's surface can restrict the effect of plasma on the vegetative cells. Scanning electron microscopic images of cells taken at 1 h post treatment shows ruptures of the cell membrane following plasma treatment (Figure 5). The accumulation of reactive species may have caused surface lesions, leading to leakage of intracellular components of the cells and eventually complete inactivation. The injured cells would not be able to recover, and death would be their next state, in the following hours. The consumption of reactive species formed during the oxidation of organic matter may explain why no significant effect was observed during storage up to 48 h in the case of black pepper. The effects of plasma treatment on microbial cells are mainly due to the plasma generated radicals (O, OH, etc.), excited or reactive molecules (O_2*, O_3, NO, etc.) or charged particles (electrons and atomic or molecular ions) and cell interactions (Critzer et al., 2007). In general, microorganisms suffer a severe bombardment by reactive species (oxygen-based reactive species ROS and nitrogen-based reactive species RNS) during plasma treatment, resulting in surface lesions on the living cells, resulting in cell death. The formation of thymer dimer due to photon emission results in distortion of the helix in microbial DNA; this could be the key microbial inactivation mechanism for cold plasma technology (Bourke et al., 2017). Several other mechanisms are proposed as responsible for

microbial inactivation in cold plasma treated foods. A reaction occurs on the double bond of ROS-sensitive unsaturated fatty acid present on the cell membrane, thus impairing the transportation of molecules in and out of the cell (Alkawareek et al., 2014; Joshi et al., 2011). In addition, UV photons also produce lethal effects on the micro-organism by altering its DNA, inhibiting DNA replication and thereby retarding cells multiplication.

3.2 Reactive species analysis

The reactive species produced by the plasma system were analysed by optical emission spectroscopy. Figure 6a-c shows the optical emission spectrum of the plasma system over the range 200 to 1100 nm. Two regions of emission can be observed: (1) the emissions due to molecular vibrations of molecular nitrogen, specifically those from the second positive system, and (2) the emissions due to atomic transitions of atomic nitrogen and oxygen. Figure 6a shows the whole spectrum and Figures 6b-c considers two narrower wavelength ranges. The region presented in Figure 6b (from 280 to 420 nm) is dominated by the nitrogen vibrational band, the second positive system, but also contains emissions from $^{\bullet}$OH and the nitrogen first negative system (highlighted in blue). The region shown in Figure 6c (from 540 to 1100 nm) is dominated by the atomic transitions from atomic nitrogen and oxygen. The spectrum shows typical characteristic lines associated with N_2, OH, H, N and O. The spectrum is dominated by molecular emissions of the N_2 second positive system ($N_2(C^3\Pi_g) \rightarrow N_2(B^3\Pi_g) + h\nu$) between the regions of 290 – 400 nm (Figure 6b). Within this region, emissions due to nitrogen molecular ions, the first negative system (${N_2}^+(B^3\Sigma_u^+) \rightarrow {N_2}^+(X^3\Sigma_u^+) + h\nu$) are also detected and highlighted in blue. Furthermore, evidence of the OH rotational transition ($OH(A^3\Sigma^+) \rightarrow OH(X^2\Pi) + h\nu$) is also seen at 308 nm. In Figure 6c atomic oxygen is seen at 615 nm $O(4d^5D \rightarrow 3p^5P)$, 777 nm $O(3p^5P \rightarrow 3s^5S)$, 844nm $O(3p^3P \rightarrow 3s^3S)$, and 925 nm $O(3d^5D \rightarrow 3p^5P)$, ionised atomic oxygen at 464 nm $O^+(3p^4D \rightarrow 3s^4P)$, and 407 nm $O^+(3d^4F \rightarrow 3p^4D)$, and ionised molecular oxygen at 559 nm $O^+(b_4\Sigma_g^- \rightarrow a_4\Pi_u^-)$and 525 nm $O^+(b_4\Sigma_g^- \rightarrow a_4\Pi_u^-)$. The Hydrogen alpha emission is seen at 656 nm ($H^*(n=3) \rightarrow H^*(n=2) + h\nu$). Lines associated with atomic nitrogen can also be observed. These lines, found at 746 nm, 821 nm, 869 nm, 939 nm and 1011 nm consist of complex multiplet peaks such as the seven peak multiplet found at 821 nm and eleven peak feature centred around 869 nm, are caused by angular momentum transitions within the nitrogen atom. The nitrogen molecular first positive system ((FPS) $N_2(B^3\Pi_g) \rightarrow N_2(A^2\Sigma_u^+)$) are also present in Figure 6.

3.3 Total phenolic content (TPC)

Table 1 shows the total phenolic content of black pepper treated by cold plasma at different conditions. No significant reduction in the phenolic content was observed among all the treated samples ($p > 0.05$) for the experimental conditions investigated. It can be concluded

that cold plasma treatment did not have any impact on phenolic content of black pepper grains. This may be due to the limited penetration depth of plasma reactive species into the black pepper as plasma is effectively a surface treatment phenomenon.

4. Conclusion

Cold plasma technology was demonstrated to have to be more effective for a model liquid system compared to black pepper grains contaminated with vegetative cells, with 4.65±0.70 log reduction compared to 2.40±0.70 log reduction for samples treated at 30 kV for 5 min and stored for 24 h at 4 ^{o}C. The SEM images showed distinguishable changes in cells' morphology after plasma treatment. Plasma treatment reduced the smoothness of the cell membrane due to lesions, eventually leading the cells' death. OES detected the presence of several reactive species (reactive oxygen species including hydroxyl radicals $^{\bullet}$OH and reactive nitrogen species including nitrite oxide NO and nitrate NO_2), as well as excited molecules (excited oxygen and nitrogen). The peaks of the spectrum measured in air reveals the presence of various oxygen-based and nitrogen-based species. These elements are considered as responsible for the microbial inactivation observed. This study demonstrates that up to 2 log reduction in *B subtilis* vegetative cells and 1 log reduction in spores contaminated black pepper grains can be achieved. The total phenolic content of black pepper revealed that plasma technology has a limited impact on the food quality.

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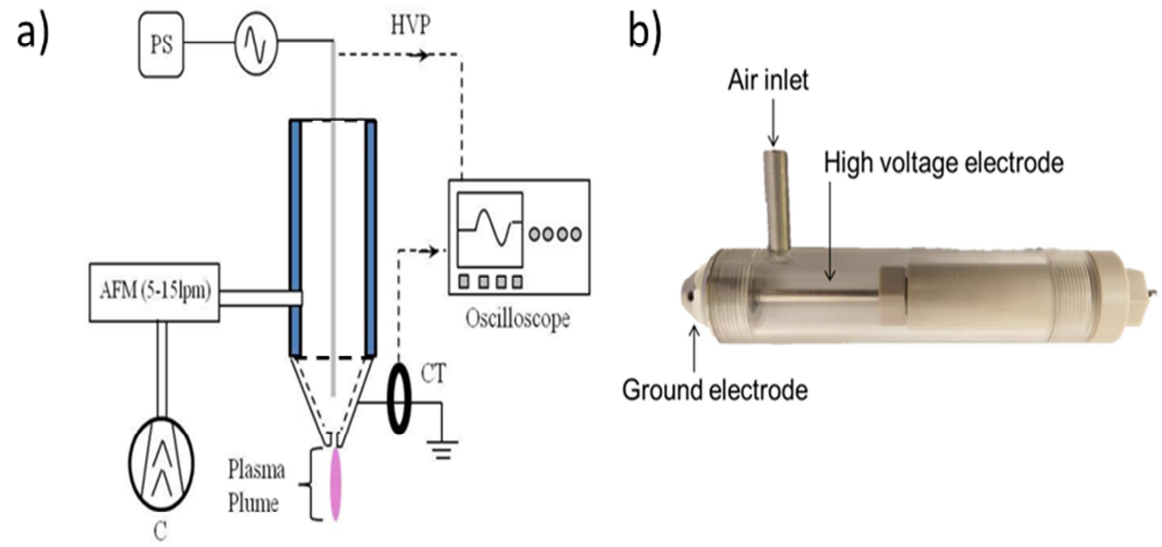


415

416 Figure 1. Experimental set up for plasma jet system a) (HVP) high voltage probe; (AFM) air
417 flow modulator; (C) gas introduction; (HPR) mass spectrometer; (CT) current probe. b)
418 image of the plasma jet.

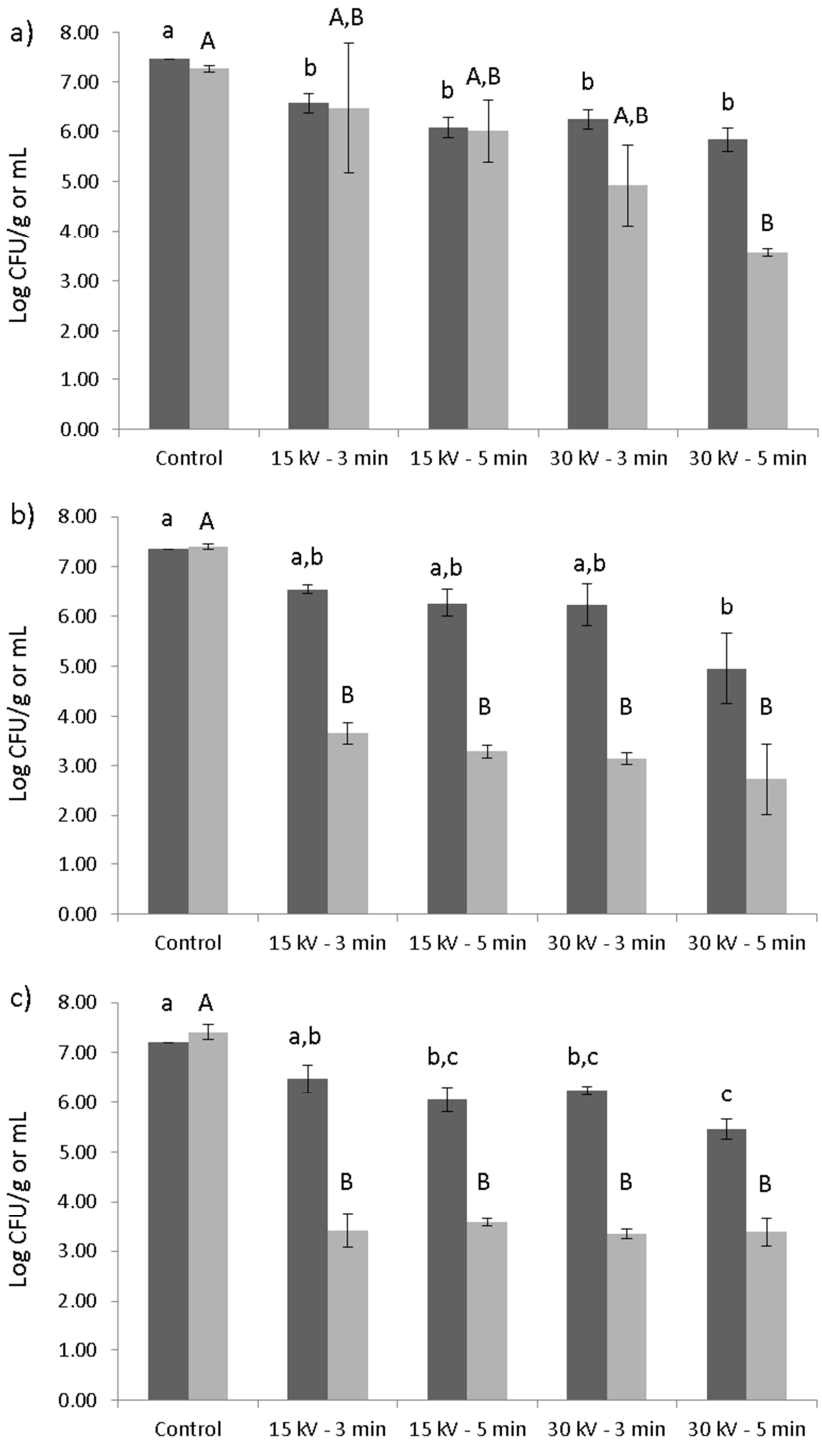


Figure 2. Inactivation of *B subtilis* vegetative cells in black pepper grains in log CFU/g (lll) and model liquid food in log CFU/mL (lll) after cold plasma treatment enumerated after 1 h (a), 24 h (b) and 48 h (c) of incubation.

[abc]Column followed by same letters are not significantly different ($p > 0.05$) for black pepper samples.

[ABC]Column followed by same letters are not significantly different ($p > 0.05$) for model liquid food samples.

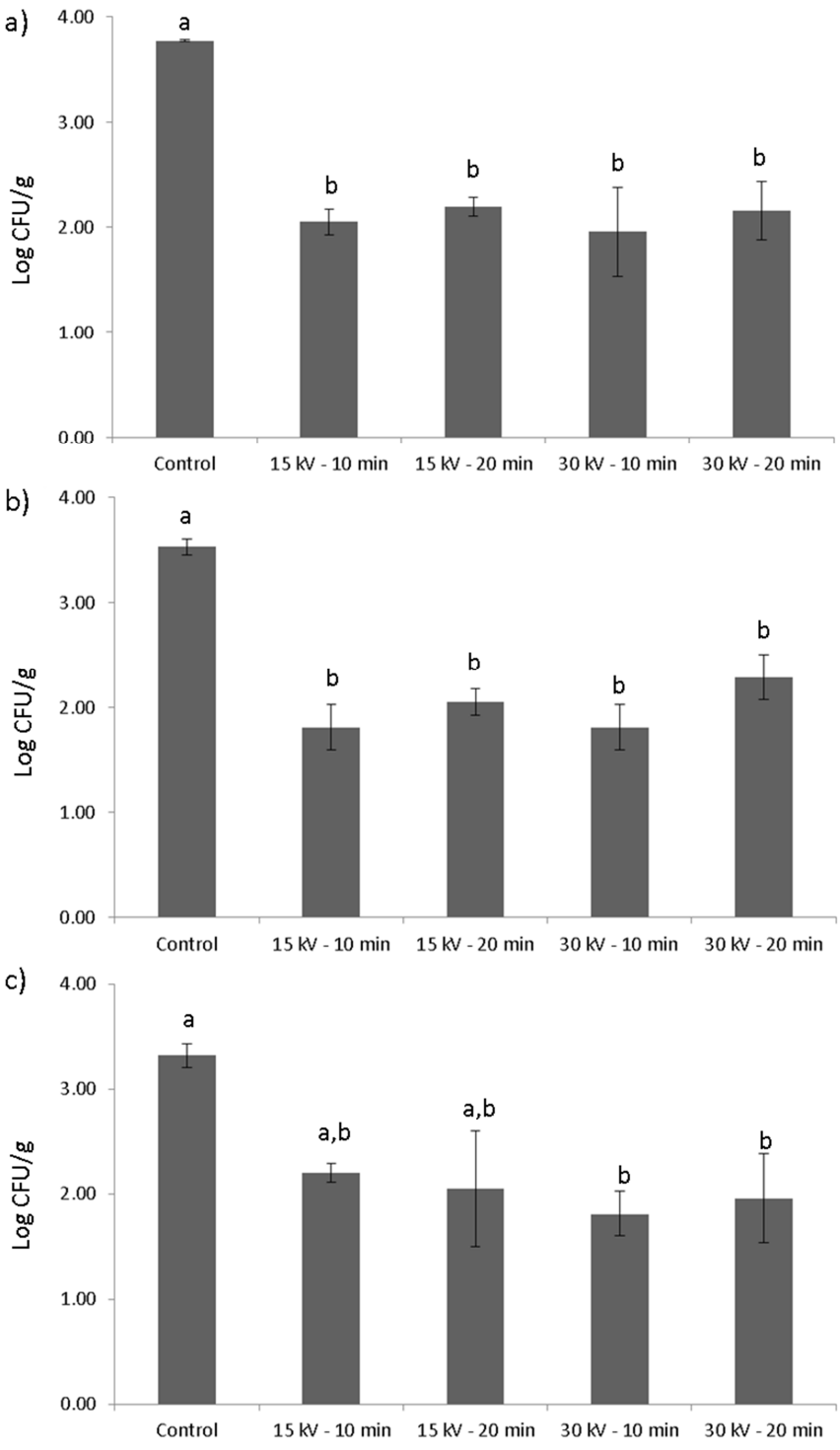


Figure 3. Inactivation of *B subtilis* spores in log CFU/g in black pepper after cold plasma treatment enumerated after 1 h (a), 24 h (b) and 48 h (c) of incubation.

[abc]Column followed by same letters are not significantly different ($p > 0.05$).

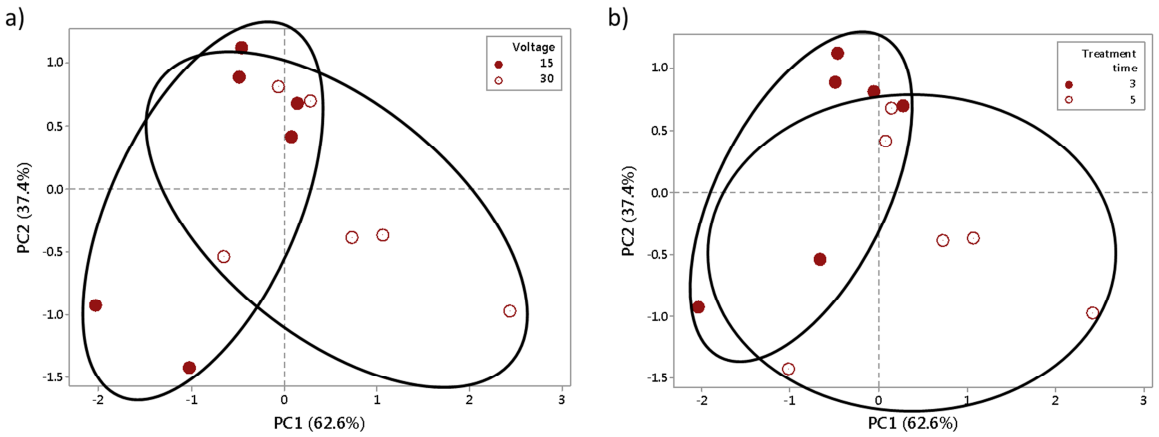
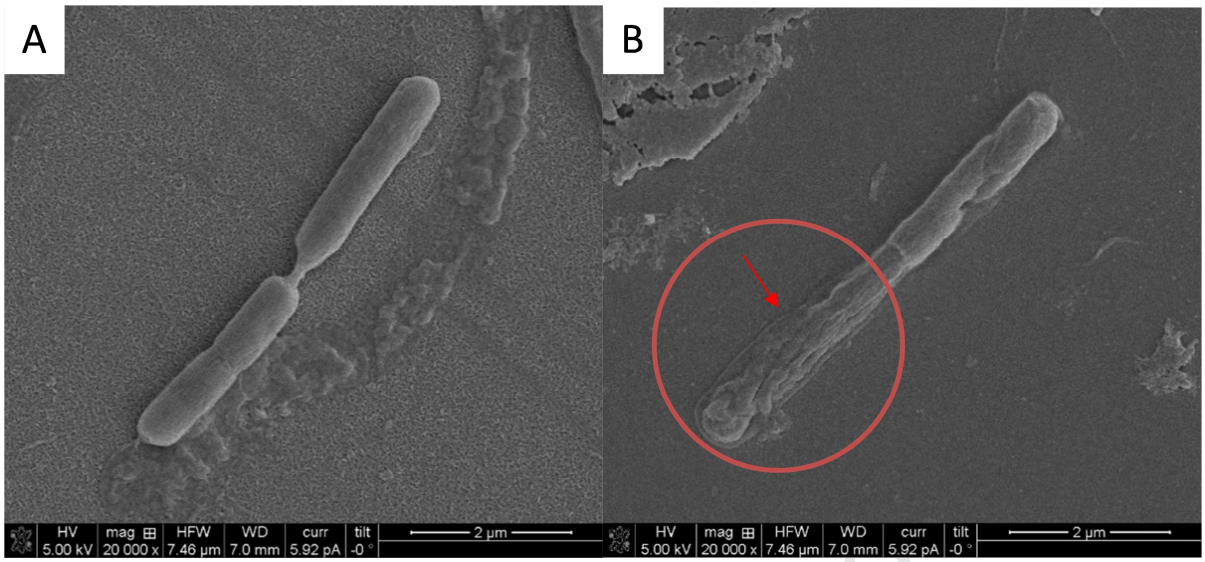


Figure 4. Score plot showing the effect of voltage in kV (a) and treatment time in min (b) for *Bacillus subtilis* vegetative cells contaminated samples.



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449 Figure 5. Scanning Electron Microscope images of *B subtilis* vegetative cells (untreated (A)
450 and after plasma treatment (B)).

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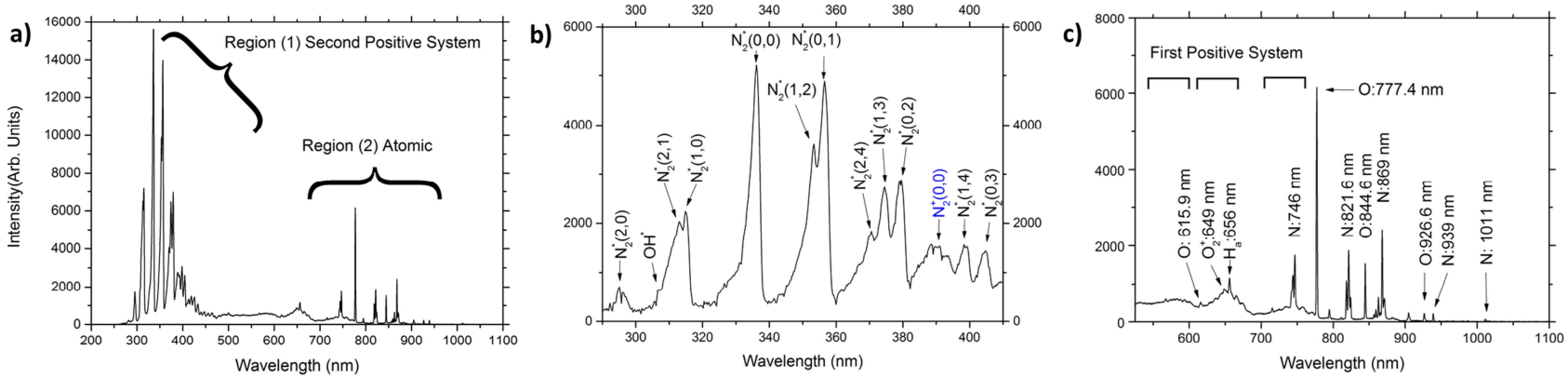

452

453 Figure 6. Optical emission spectrum of the cold plasma system from 200 to 1100 nm (a), 280 to 420 nm (b) and 540 to 1100 nm (c).

454 Table 1. Changes in total phenolic content (TPC, mg GAE/g) subjected to plasma treatment at 1 h, 24 h and 48 h post treatment.

455

Treatments	Total Phenolic Content (mg GAE/g)		
	(+1 h)	(+24 h)	(+48 h)
Control	2.26±0.45[a]	2.13±0.68[a]	1.96±0.52[a]
15 kV - 3 min	2.36±0.33[a]	2.13±0.57[a]	1.91±0.81[a]
15 kV - 5 min	2.51±0.69[a]	2.18±0.75[a]	1.98±0.52[a]
30 kV - 3 min	2.15±0.57[a]	2.22±0.75[a]	1.80±0.64[a]
30 kV - 5 min	2.34±0.45[a]	2.09±0.41[a]	1.98±0.73[a]

456 [a]Values followed by same letters are not significantly different ($p > 0.05$).

457

Highlights

- Application of cold plasma technology for microbial inactivation
- Analysis of reactive species generated by plasma using optical emission spectroscopy
- Spoilage microorganisms can be inactivated by cold plasma
- No significant degradation of key quality parameters observed for dried ingredients

CONFLICT OF INTEREST

Declarations of interest for the manuscript "EFFECT OF NON-THERMAL PLASMA TECHNOLOGY ON MICROBIAL INACTIVATION AND TOTAL PHENOLIC CONTENT OF MODEL LIQUID FOOD AND BLACK PEPPER GRAINS" submitted to Lebensmittel-Wissenschaft & Technologie - Food Science & Technology Journal: **none**.

Yours sincerely,

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